Spectrin/Actin Complex Isolated from Sheep Erythrocytes Accelerates Actin Polymerization by Simple Nucleation

EVIDENCE FOR OLIGOMERIC ACTIN IN THE ERYTHROCYTE CYTOSKELETON*

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*A complex of spectrin and actin, isolated from sheep erythrocyte ghosts, accelerates the polymerization of actin in buffer containing 0.3 mm MgCl₂. The rate of actin polymerization is similarly increased by sonicated F-actin nuclei. At steady state, the critical concentration of actin is lower when polymerization occurs in the presence of spectrin/actin complex than in its absence. Polymerization of actin in the presence of spectrin/actin complex is inhibited by substoichiometric concentrations of cytochalasin D, which is thought to block the net polymerizing ends of growing actin filaments (Brenner, S. L., and Korn, E. D. (1979) J. Biol. Chem. 254, 9982-9985), in the same way that cytochalasin D inhibits actin polymerization in the absence of complex. However, actin filaments formed in the presence of complex are more stable to cytochalasin D added after polymerization has occurred than are filaments formed in the absence of complex. We conclude from these data that spectrin/actin complex accelerates actin polymerization by simple nucleation and, therefore, that the complex consists of short actin oligomers cross-linked by spectrin tetramers (Brenner, S. L., and Korn, E. D. (1979) J. Biol. Chem. 254, 8620-8627) which stabilize the net depolymerizing ends of the actin oligomers. The isolated spectrin/actin complex is a fragment of the erythrocyte cytoskeleton. From our proposal for the structure of the complex and estimates of others for the relative amounts of actin, spectrin, and spectrin/actin complex-related high affinity cytochalasin binding sites in human erythrocyte ghosts, we suggest that the cytoskeletal network contains short oligomers of actin consisting on average of about ten subunits each, with one of every two actin subunits cross-linked by a spectrin tetramer to a subunit of another actin oligomer.

The isolated erythrocyte ghost consists of the plasma membrane and an underlying cytoskeleton that is thought to be the primary determinant of erythrocyte shape and deformability in situ (1, 2). The major constituents of the cytoskeleton are spectrin (bands 1 and 2, according to the nomenclature of Fairbanks et al. (3)), band 4.1, and actin (band 5) in the approximate molar ratio of 1:1:2 (4). Spectrin is a heterodimer of a 240,000-dalton polypeptide and a 220,000-dalton phosphorylated polypeptide (3, 5) that further associates to a tetrameric species (6) believed to be the native form of the protein in the erythrocyte (6, 7). Band 4.1 has a molecular weight of about 78,000 and band of about 42,000. The cytoskeleton is linked to the plasma membrane apparently through band 2.1, a protein of molecular weight about 210,000 that possesses binding sites both for spectrin (8, 9), and an integral membrane protein that migrates electrophoretically with band 3 (10). Until very recently, however, there has been almost no evidence for the nature of the interactions among the three major cytoskeletal proteins, spectrin, band 4.1, and actin.

The first important step in this regard was the suggestion by Pinder et al. (11, 12), derived from experiments with partially purified low ionic strength extracts of human erythrocyte ghosts, that spectrin could induce the polymerization of G-actin to F-actin. After Ungewickell and Gratzer (6) and Rabston (13) showed that the spectrin in crude extracts of human erythrocyte ghosts can be separated by gel chromatography into three fractions, a spectrin/actin/band 4.1 complex, spectrin tetramer, and spectrin dimer, we found that only the spectrin/actin complex isolated from sheep erythrocyte ghosts (this complex does not contain detectable levels of band 4.1) has the ability to accelerate the polymerization of actin (14). We found no evidence for the interaction of spectrin dimer or spectrin tetramer with monomeric G-actin. Spectrin dimer does bind to F-actin, however, and spectrin tetramer, irrespective of its state of phosphorylation, cross-links F-actin into highly viscous gels (14).

From these data, from the relative amounts of spectrin and actin in erythrocyte ghosts, and from the apparent absence of detectable actin filaments in ghosts (15), we proposed (14) that the erythrocyte cytoskeleton consists, in part, of short actin oligomers cross-linked by spectrin tetramers. On this assumption, the spectrin/actin complex isolated from the low ionic strength extract of sheep erythrocyte ghosts could be viewed as a partial fragment, missing band 4.1, of the erythrocyte cytoskeleton. Before considering how such a complex might affect the polymerization of actin in vitro, and what biological significance to attribute to this phenomenon, it is necessary to summarize briefly the properties of actin polymerization as presently understood (16-18).

The rate-determining step in the polymerization of G-actin to F-actin is the condensation of several actin monomers to form an actin nucleus. Under polymerizing conditions, actin monomers probably associate and dissociate from both ends of the nucleus but with different rate constants at the two ends (18). Normally, under optimal polymerizing conditions, 1 molecule of ATP is hydrolyzed for each molecule of actin monomer added to the growing filament. Polymerization will cease, and the ratio of polymer to monomer will reach a steady state, when the net rate of addition of monomers equals the net rate of loss of monomers. When ATP is present, Wegner has shown (18) that each end of the filament will have its own critical concentration, i.e. the concentration of monomer in
equilibrium with polymer. Therefore, neither end will be in equilibrium when the system as a whole is at steady state and exceeds the net rate of addition of actin monomers. Further, during polymerization or depolymerization, conditions could exist in which net addition or net loss of monomers occurs at both ends of the actin filament simultaneously, i.e., the filament could have two net polymerizing ends or two net depolymerizing ends.

If the spectrin/actin complex were, in fact, a fragment of the erythrocyte cytoskeleton consisting of oligomeric actin cross-linked by spectrin tetramer, it would be expected to have the effect on actin polymerization first described by Pinder et al. (11, 12) by providing stable actin nuclei with accessible net polymerizing ends. The experiments described in this paper were designed to test this hypothesis. While they were in progress, a paper was published by Lin and Lin (18) which had an important influence on some of our experiments.

**MATERIALS AND METHODS**

Muscle actin was prepared from rabbit back and leg muscles by a modification (20) of the method of Spudich and Watt (21). Acanthamoeba profilin was prepared according to Reichstein and Korn (22). ATP and phenylmethanesulfonyl fluoride were purchased from Sigma, Tris from Bethesda Research Laboratories, reagents for polyacrylamide gel electrophoresis from Bio-Rad, and cytochalasins B, D, and E from Aldrich. Stock solutions of 10 mM cytochalasins in dimethyl sulfoxide were stored at 4°C and diluted with dimethyl sulfoxide as required to maintain 1% dimethyl sulfoxide in the experimental solutions while varying the concentrations of the cytochalasins. All other chemicals were at least reagent grade and deionized water was used.

Purified actin was quantified spectrophotometrically using extinction coefficients at 290 nm of 617 cm/g and 638 cm/g for G-actin and F-actin, respectively (23). Spectrin/actin complex and profilin were quantified by the method of Lowry et al. (24) with bovine serum albumin (Armour) as standard. Dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Brenner and Korn (14). Samples for viscometry were mixed on ice and 0.5 ml was transferred to Cannon-Manning semi-microviscometers (size 100) at 30°C and the flow times of the samples were measured. Specific viscosities were calculated as follows: (sample flow time — buffer flow time)/(buffer flow time). The buffer flow time was about 60 to 70 s.

**RESULTS**

**Effect of Spectrin/Actin Complex on Actin Polymerization**—As measured by the increase in viscosity, polymerization of actin at 30°C is complete within 5 to 10 min when 2 mM MgCl₂ is added to G-actin in 5 mM Tris-chloride, pH 8.0, 0.2 mM ATP, 0.2 mM dithiothreitol, and 0.1 mM CaCl₂ (17). It requires 25 min to attain 50% polymerization in 0.5 mM MgCl₂ and 50 min for 50% polymerization in 0.4 mM MgCl₂, while in 0.3 mM MgCl₂, polymerization is usually less than 75% complete after 60 min (17). A few of our actin preparations polymerize less well and behave in 0.4 mM MgCl₂ as most preparations do in 0.3 mM MgCl₂. Since all the solutions contain 0.2 mM ATP, the free Mg⁺ concentration is about 0.2 mM less than the total Mg²⁺ concentration.

The data in Fig. 1 demonstrate that polymerization of actin in the presence of 0.3 mM MgCl₂ is greatly accelerated by addition of small amounts of spectrin/actin complex from sheep erythrocytes. In the presence of 72 μg/ml of complex, the rate and extent of polymerization of 20 μM actin in 0.3 mM MgCl₂ is essentially the same as is obtained in 2 mM MgCl₂ (Fig. 1). This is the same observation previously reported for the polymerization of actin in 10 mM phosphate (11, 12, 14) or in 0.4 mM MgCl₂ (19) and interpreted as induction of polymerization in nonpolymerizing buffers. In fact, however, actin alone does polymerize, although slowly, in 0.3 mM MgCl₂ and, if polymerization is allowed to proceed to completion, similar steady state viscosities are obtained in the absence and in the presence of spectrin/actin complex (Table 1).

The rate of actin polymerization depends on the concentration of actin nuclei. Since the only compositional difference between spectrin/actin complex and spectrin dimer or tetramer, neither of which has any effect on the rate of polymerization, was omitted from Ref. 14. The figure can be found in the “Additions and Corrections” section of J. Biol. Chem. (1979) 254, 12738 and corrected reprints are available from the authors.

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1 Due to a printer's error, the relevant figure (Fig. 1A) was omitted from Ref. 14. The figure can be found in the "Additions and Corrections" section of J. Biol. Chem. (1979) 254, 12738 and corrected reprints are available from the authors.
zation of actin (14), is the presence of actin in the complex (Fig. 2), it seemed likely that spectrin/actin complex accelerates actin polymerization by providing a source of net polymerizing ends to which actin monomers can add. As would be expected if this were the case, the effect of spectrin/actin complex can be duplicated by addition of sonicated F-actin nuclei (Fig. 3): 0.04 mg/ml (1 µM) of sonicated F-actin produces essentially the same rate of polymerization of 20 µM G-actin as is obtained by 0.05 mg/ml of spectrin/actin complex and about the same rate of polymerization as is obtained by adding 2 mM MgCl₂. The steady state viscosity in 0.4 mM MgCl₂, however, is lower in the absence of spectrin/actin complex for reasons that are discussed later. Although they are qualita-

**Table 1**

**Steady state viscosities of actin at low ionic strength in the presence and absence of spectrin/actin complex**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Specific viscosity</th>
</tr>
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<tbody>
<tr>
<td>Actin + complex</td>
<td>0.02</td>
</tr>
<tr>
<td>Complex + MgCl₂</td>
<td>0.02</td>
</tr>
<tr>
<td>Actin + MgCl₂</td>
<td>0.77</td>
</tr>
<tr>
<td>Actin + complex + MgCl₂</td>
<td>0.80</td>
</tr>
</tbody>
</table>

**Fig. 3. Effect of sonicated F-actin nuclei on actin polymerization.** Sonicated F-actin nuclei were added to 20 µM G-actin at 0°C immediately after addition of 0.4 mM MgCl₂ to buffer containing 5 mM Tris-chloride, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM CaCl₂, 0.01% NaN₃, 1% dimethyl sulfoxide, 7.8% sucrose, pH 8.0. Samples were incubated at 30°C for 19 h and transferred to viscometers for measurement of their steady state viscosities. Each number is the average of at least three measurements on the same sample.

![Complex and Spectrin](image)

**Fig. 2. Dodecyl sulfate-polyacrylamide gel electrophoresis of spectrin/actin complex and spectrin tetramer.** Spectrin/actin complex (15 µg) and spectrin tetramer (10 µg) were applied to 7.5% polyacrylamide slab gels. Spectrin (bands 1 and 2) and actin (band 5) are labeled. Complex was prepared as in Ref. 14. The molar ratio of spectrin dimer to actin monomer in the complex is 1:2 by scans of the negative.
should be lower in the presence of spectrin/actin complex the measured critical concentration for actin polymerization than in its absence. In the experiment described in Fig. 4, otherwise have occurred. Complex should reduce the critical concentration at the net depolymerizing ends and, therefore, the measured critical concentration for actin polymerization should be lower in the presence of spectrin/actin complex than in its absence. In the experiment described in Fig. 4, the presence of spectrin/actin complex did reduce the critical concentration (the actin concentration at zero specific viscosity) from the control value of 4.5 μM to about 0.2 μM. If all of the filaments at steady state in the presence of complex were associated with complex, the measured critical concentration of 0.2 μM would be the critical concentration, i.e., the reciprocal of the equilibrium constant for association, for the net depolymerizing ends of actin filaments.

**Effect of Cytochalasins on the Polymerization of Actin in the Presence and Absence of Spectrin/Actin Complex**—While the experiments described in this paper were in progress, Lin and Lin (19) discovered that the high affinity cytochalasin binding sites of human erythrocytes that are not involved in sugar transport are associated with the spectrin/actin/band 4.1 complex and that cytochalasins inhibit the ability of that complex to accelerate the polymerization of actin. In complete agreement with Lin and Lin (19), we find (Fig. 5) that substoichiometric concentrations of cytochalasin D (0.2 μM) inhibit the polymerization of actin (20 μM) in the presence of sheep erythrocyte spectrin/actin complex (55 μg/ml). As reported by Lin and Lin (19), cytochalasin B is only about one-tenth as effective an inhibitor of actin polymerization as is cytochalasin D (Fig. 5).

From the facts that the cytochalasin-inhibited complex from sheep erythrocytes contains only spectrin and actin (Figs. 2), that pure spectrin does not bind cytochalasins (19) nor detectably affects actin polymerization (14), and that the effects of complex on actin polymerization can be duplicated by sonicated F-actin nuclei (Fig. 3), we deduced that the cytochalasins probably interact specifically with stabilized actin oligomers in the spectrin/actin complex. Cytochalasins, therefore, should also effectively inhibit actin polymerization in the absence of complex. We were able to show (17) that substoichiometric concentrations of cytochalasin D inhibit the polymerization of actin in the absence of spectrin/actin complex just as in its presence. These data led to the hypothesis (17) that cytochalasins act primarily by blocking the net polymerizing ends of actin nuclei and filaments. One demonstrable consequence of that interaction is that addition of cytochalasin D to actin filaments, in the absence of spectrin/actin complex just as in its presence.
actin complex, caused a rapid decrease in viscosity to the same low viscosity that was attained when cytochalasin D has been present from the beginning of the polymerization (17). This would result from cytochalasin blocking the net depolymerizing ends of the growing actin filaments while allowing the net depolymerizing ends to reach their independent equilibrium with the pool of monomeric actin.

If spectrin/actin complex does, as we propose, block the net depolymerizing ends of growing actin filaments, we would expect a different result when cytochalasin D is added to actin filaments polymerizing in the presence of complex than was obtained in the absence of complex. Cytochalasin should, as before, inhibit further filament growth but filaments attached to complex should not depolymerize.

Experimentally, addition of 0.2 μM cytochalasin D, a concentration that strongly inhibits actin polymerization, at various times after initiation of actin polymerization in the presence of spectrin/actin complex, immediately stops further polymerization (Fig. 6) as predicted. Moreover, the viscosity does not fall to the very low level observed when cytochalasin D is present from the beginning of the incubation, as it does when the same experiment is carried out in the absence of complex (17). The small decrease in viscosity that occurs, especially when cytochalasin is added near the end of polymerization, is probably due to the presence of actin filaments that are not attached to complex. Such unattached actin filaments could arise either from slow self-nucleation of polymerization, as occurs under these conditions in the absence of complex (Figs. 1, 3, 4, 7, and Table I), or from dissociation of filaments from complex subsequent to their formation.

Effect of Profilin on the Polymerization of Actin in the Presence of Spectrin/Actin Complex—Profilin is a low molecular weight protein that inhibits actin polymerization (22, 23) by forming a 1:1 complex with monomeric actin. Under the conditions of polymerization used in this paper, spectrin/actin complex largely counteracts the inhibition by profilin (Fig. 7) just as actin nuclei were previously shown to do (22). This is additional evidence for the qualitative similarity of the polymerization processes in the presence and absence of complex.

**DISCUSSION**

We have demonstrated in this paper that spectrin/actin complex isolated from sheep erythrocyte ghosts accelerates the rate of actin polymerization and lowers the steady state critical actin concentration under conditions in which actin alone will also polymerize, albeit more slowly than in the presence of complex. The kinetic effect of spectrin/actin complex is mimicked by sonicated F-actin nuclei. Cytochalasins inhibit complex-accelerated actin polymerization and interact with actin filaments formed in the presence of complex in a manner generally predictable from their effects on actin polymerization in the absence of complex (17). The results reported in this paper were all obtained in 5 mM Tris-chloride containing 0.3 or 0.4 mM MgCl₂, a buffer similar to that used by Lin and Lin (19), but identical results have also been obtained in 10 mM phosphate, the buffer used by Pinder et al. (41, 12).

We believe, on the basis of these observations, that there is no need to attribute any unique properties to the spectrin/actin complex. The data are consistent with the simple proposal that the spectrin/actin complex consists of short actin oligomers cross-linked by spectrin tetramers (14). Complex would thus provide stabilized actin nuclei with accessible net polymerizing ends and blocked net depolymerizing ends. In fact, under the conditions of the isolation of complex, both ends of the actin oligomers might be net depolymerizing ends and the oligomers may survive only because depolymerization is blocked at both ends by cross-linking spectrin tetramers. Cytochalasin would inhibit complex-accelerated actin polymerization, as it does actin polymerization in the absence of complex, by blocking net polymerizing ends under otherwise polymerizing conditions. Little depolymerization of actin filaments occurs when cytochalasin is added to filaments formed in the presence of complex because, even at steady state, the net depolymerizing ends of many of the actin filaments are blocked by their association with spectrin/actin complex. This model for the structure of the spectrin/actin complex, and the effect of complex and the cytochalasins on actin polymerization, is schematically illustrated in Fig. 8.

The isolated spectrin/actin complex is most simply considered as a partial fragment of the erythrocyte cytoskeleton. The possible size of actin oligomers in the erythrocyte can then be estimated from the above model and the further assumption that each actin oligomer has one free end accessible to cytochalasins. Steck (4) has estimated that each human erythrocyte ghost contains about 3.5 × 10⁶ actin monomers and Lin and Lin (19) have estimated that the spectrin/actin/band 4.1 complex accounts for about 3 to 4 × 10⁶ high affinity cytochalasin binding sites in each erythrocyte ghost. On this basis, the average actin oligomer would contain about 10 actin monomers and be only about 26 nm long (28), if the actin oligomer is a typical double helical actin structure. The actin oligomer, therefore, would be considerably shorter than the maximum length of just one spectrin tetramer, which is estimated to be about 200 nm long (27). From the reported molar ratio of spectrin to actin in human erythrocyte ghosts (4), approximately one of every two monomers in each actin oligomer would be cross-linked by a spectrin tetramer to a subunit of another actin oligomer in a continuous cytoskeletal network (Fig. 9, top). This model is compatible with the continuous, web-like reticulum of filaments 5 to 40 nm in diameter and 20 to 100 nm in length revealed by scanning electron microscopy (15).
electron microscopy of the cytoplasmic surface of lysed erythrocyte membranes (28); the narrower filaments could be actin oligomers and the wider filaments might be spectrin tetramers.

In addition to spectrin and actin, the complexes isolated from human erythrocytes by Lin and Lin (19) and Cohen and Branton (29) also contain band 4.1. Although band 4.1 is apparently not necessary for the acceleration of actin polymerization by the complex, nor for the inhibition of that effect by cytochalasins, band 4.1 is probably an important structural element of the erythrocyte cytoskeleton. Possibly, band 4.1, which is present in approximately molar equivalence to spectrin (4), provides stability to the cytoskeleton by cross-linking spectrin to actin near the sites of their interaction. Another protein, band 4.2, that is believed to interact with this system (30), may be associated (8, 10) with the linkage of the cytoskeleton to the plasma membrane through band 2.1 (Fig. 9, bottom).

Finally, we attribute no particular biological significance to the ability of isolated spectrin/actin complex to accelerate the polymerization of actin. Rather, it seems to be just a fortuitous consequence of the structure of the erythrocyte cytoskeleton from which the complex is derived. Cohen and Branton (29) have reached a similar conclusion from their observation that the orientation of the newly polymerized actin filaments associated with complex in situ is the opposite of that generally found for membrane-associated filaments in vitro. However, it should be remembered that, in practice as well as in theory, conditions can be found in which either or both (31) ends of an actin filament can be net polymerizing ends. Nonetheless we would expect that any complex of actin filaments cross-linked and stabilized by any actin-binding protein, irrespective of its possible membrane association or function in situ would, when isolated from cells, have properties similar to those of the spectrin/actin complex isolated from erythrocytes, i.e. the ability to bind cytochalasins and to nucleate cytochalasin-inhibitable actin polymerization.

Addendum—After this paper was accepted for publication, a paper appeared (32) with evidence that band 4.1 may be required to form stable complexes between spectrin tetramer and F-actin. That is not incompatible with the data in this paper and Ref. 14 for direct interaction between spectrin and actin and agrees with our speculation that spectrin/actin interactions in situ might be stabilized by cross-links of band 4.1.

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